Chapter 1

General introduction and scope of this thesis

Bacillus subtilis

Bacillus subtilis is a Gram-positive, rod-shaped, endospore-forming bacterium, characterized by its robustness and high adaptability to changing environmental conditions. Consequently, *B. subtilis* has become a widely distributed organism in nature[1]. In fact, it can be found in niches that include the upper layers of the soil, aquatic environments (rain, or groundwater)[2], and even the gut of mammals including humans[3,4]. *B. subtilis* can grow in such diverse environments because it can withstand wide variations in temperature (11-52°C)[5,6] and pH (6-9)[7–9]. Under these conditions, it is able to secrete a vast number of enzymes and antimicrobial peptides into the extracellular space, allowing this bacterium to outcompete other microorganisms[10,11].

In addition to its success in nature, *B. subtilis* has also become a success in industry, especially for the biotechnological production of enzymes. This relates to this bacterium's superior protein secretion levels, its genetic amenability, its robustness in the fermentation process, and the lack of exotoxins and endotoxins. While the high-level secretion of enzymes into the fermentation broth makes their downstream processing cost-effective, the lack of toxins in the fermentation process has earned many *B. subtilis* products the Generally Recognized As Safe (GRAS) status from the US Food and Drug Authority (FDA). Due to these features, *B. subtilis* has become one of the most important production platforms in the detergent, beverage, food, animal feed, leather, and chemical industries[10–14]. Approximately 60% of commercial enzymes are currently produced in *Bacillus* species[15]. Moreover, *B. subtilis* can also produce bioproducts, such as riboflavin and hyaluronic acid[16]. Advances in molecular biology and systems biology have enabled the continuous development of industrial *B. subtilis* strains as they are being used in the industry today.

Despite the excellent performance in enzyme production, high-level heterologous protein expression frequently encounters secretion bottlenecks. In the ideal scenario, secretory expression guarantees that recombinant proteins synthesized in the cytoplasm successfully reach the extracellular medium, with minimal protein misfolding, aggregation, and degradation. Importantly, faulty protein secretion can have deleterious effects on the host cell's viability and its productivity[17,18]. Therefore, a deeper understanding of the physiological consequences of protein secretion by *B. subtilis* is of key importance for the design and engineering of next-generation super-secreting strains that can produce a broad spectrum of biotechnologically and bio-pharmaceutically relevant proteins.

Protein secretion in *B. subtilis*

B. subtilis has the ability to transport approximately 300 proteins from the cytoplasm to the membrane, cell wall, and extracellular space[19,20]. This bacterium employs two main mechanisms to translocate proteins across the membrane[21]: the general secretory (Sec) pathway and the twin-arginine (Tat) pathway (**Fig 1**).

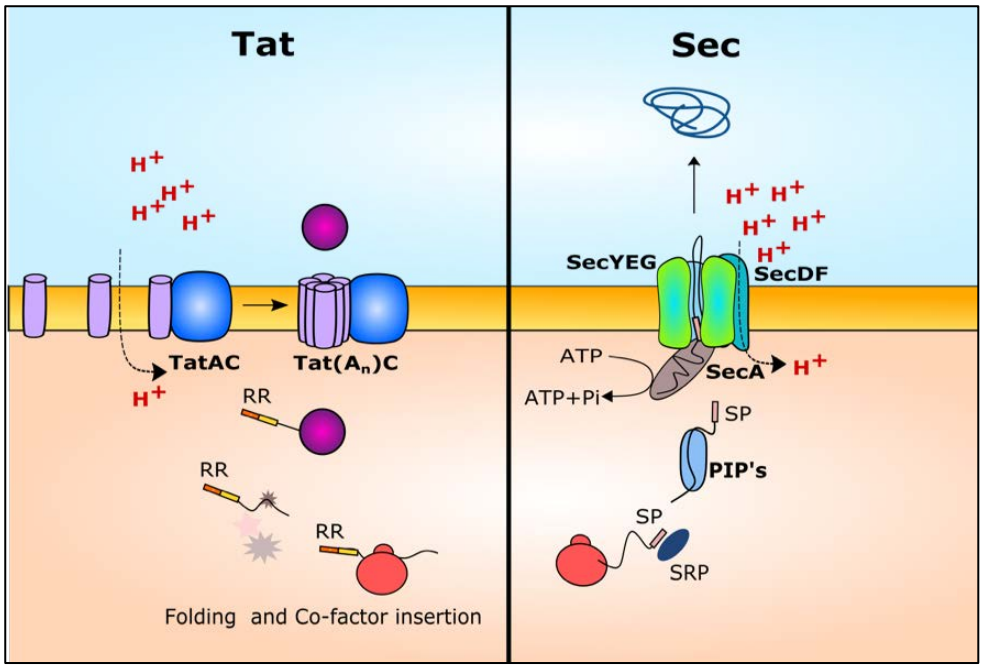

Fig. 1: The main mechanisms of protein translocation in *B. subtilis*. Proteins transported by the Twin-arginine (Tat) pathway are characterized by a twin-arginine (RR) motif in their signal peptide. After translation, Tat substrates are folded and a cognate co-factor is usually inserted into each protein. Subsequently, folded proteins interact with the receptor complex TatAC, leading to the oligomerization of multiple TatA proteins in a proton-motive force (pmf)-dependent mechanism. This results most likely in a local weakening of the membrane, which enables passage of the Tat substrate through the membrane. In contrast, the general secretory pathway (Sec) translocates proteins across the membrane in an unfolded state. Pre-proteins with a proper Sec-type signal peptide may interact with the so-called signal recognition particle (SRP). Furthermore, Sec substrates can be maintained in an unfolded state with the help of post-translationally interacting proteins (PIPs). To initiate membrane translocation, the Sec substrates interact with a SecA dimer. SecA binds to a membrane-embedded protein-conducting channel composed of SecY, SecE, and SecG, through which the Sec substrate is translocated. In addition, SecDF employs the transmembrane pmf as an energy source to drive protein translocation. Adapted from[22].

The Sec pathway

The Sec pathway transports most of the proteins that need to reach the extracellular space or that need to be inserted into the cytoplasmic membrane[23]. Consequently, the Sec pathway has been extensively employed for the production of most commercial enzymes in *B. subtilis,* such as α-amylases and proteases[13]. The Sec pathway is characterized by the transport of substrates in an unfolded state through a narrow pore in the membrane, which is formed by

the proteins SecY, SecE and SecG[24]. Sec substrates are characterized by the presence of a signal peptide that consists of a positively charged N-terminal region, followed by a hydrophobic H-region, and a polar carboxyl-terminal C-region that contains a consensus amino acid sequence motif (Ala-X-Ala) necessary for recognition and cleavage by signal peptidase[22]. For the translocation process to occur, Sec substrates with the proper signal peptides may be recognized already in the ribosome by the so-called signal recognition particle (SRP), forming a SRP-ribosome-pre-protein complex. This complex interacts with the SRP receptor FtsY, which transfers the pre-protein to the SecYEG channel. In the post-transcriptional Sec-dependent translocation, chaperones help the pre-protein to maintain an unfolded state. The signal peptide is subsequently recognized by SecA, an ATP-driven motor protein that pushes the pre-protein through the translocation channel[25,26]. Subsequently, signal peptidases cleave off the signal peptide and the mature protein is released for folding or further processing[26]. Other proteins involved in this process include the membrane proteins SecD and SecF, or a natural SecDF fusion protein[27], which help in the release of mature peptide[28]. Notably, the early stages in Sec-dependent protein secretion in *B. subtilis* are still not very well understood. By analogy to the Sec pathway in other organisms, like *Escherichia coli*, it seems most likely that the SRP and FtsY are mostly involved in membrane protein biogenesis. Also, the nature of post-translationally interacting proteins (PIPs) that serve to keep pre-proteins of *B. subtilis* in an unfolded state prior to membrane translocation by Sec is presently unclear. On the contrary, factors involved in the post-translocational folding and quality control of Sec-translocated proteins have been studied in great detail[19,29].

The Tat pathway

The Tat pathway enables the translocation of fully folded proteins across the cytoplasmic membrane and the biogenesis of specific integral membrane proteins[30–32] by employing the transmembrane proton motive force (pmf) as a driving force[33]. The Tat pathway first received its designation due to the presence of two adjacent “twin” arginine residues (RR) in the signal peptide of its substrates[34]. Proteins transported by the Tat pathway display a unique set of features that sets them apart from Sec substrates. First, the Tat substrates contain specific signal peptides that conform to the universally conserved tripartite signal peptide structure, but differ from Sec-type signal peptides in: (i) the positively charged N-terminal domain containing the characteristic twin-arginine amino acid sequence motif (S/T-RR-x-FLK), and (ii) a central H-domain with low hydrophobicity compared to the H-domain of Sec-type signal

peptides[30,35]. The lower overall hydrophobicity of the Tat signal peptides is hypothesized to be less likely to destabilize the substrate protein[36,37]. Second, the Tat-dependently translocated proteins often contain complex cofactors (e.g. iron-sulfur clusters, haem groups), simple metal ions (e.g. zinc, manganese), or nucleotides[38]. These cofactors are bound by the Tat substrates already in the cytoplasm. This requires the proper folding of the respective proteins prior to membrane translocation, which is in fact one of the hallmarks of Tat-dependent protein translocation[39,40]. Third, since the Tat substrates must be properly folded and have attained their native conformation prior to secretion, they are subject to strict quality control by the Tat machinery[40]. Lastly, the known Tat substrates are highly variable in size, ranging from 18-150 kDa, which implies a high conformational plasticity of the Tat translocase[41–43]. Interestingly, various mutant Tat variants have been identified that enable the translocation of misfolded proteins that cannot be transported by the wild-type Tat translocase. These mutations overrule the quality-control mechanism, demonstrating that the Tat translocases actively participate in the proofreading of their substrates[44]. The mechanism of this proofreading is currently not fully understood. However, the available data imply that the Tat pathway prefers to transport rigid structures. The sensing of conformational flexibility in a substrate will usually lead to its rejection by the Tat translocase[45].

The Tat translocase

Translocation by the Tat pathway takes place via complexes formed by integral membrane proteins belonging to two different structural families, TatA and TatC. TatA is a small L-shaped protein characterized by a short N-terminal transmembrane domain that does not fully span the membrane, followed by a cytoplasmic amphipathic helix that lies at the membrane and an unstructured C-terminal tail[46,47]. In contrast, TatC is substantially larger than TatA. TatC is characterized by having 6 transmembrane domains and its conformation is reminiscent of that of a cupped hand with a deep groove in the center, where signal peptides are accommodated[48,49].

Minimal Tat systems only contain TatA and TatC proteins, and they are usually found in the Firmicutes, such as *B. subtilis*[19,50]. However, most organisms such as plants, Gram-negative bacteria, and archaea have a second TatA-like protein, known as TatB, which evolved from a genomic duplication of the *tatA* gene[50,51]. In addition, some organisms, have yet a third TatA paralogue, typified by *E. coli* TatE. This additional third TatA paralogue is not essential for the

translocation process, unlike the TatA and TatB proteins[34]. Of note, most biochemical and physiological studies on the Tat pathway have been done in *E. coli*, or in other organisms with TatABC-type translocases[48,50]. The Tat system of *B. subtilis* represents the best-studied minimal TatA-TatC pathway[32,52,53].

The Tat translocation processes

In the first step in the translocation process, TatC interacts with TatB to form the core receptor complex (**Fig. 2**). Multiple studies have suggested that TatA and TatE are present to some degree in this complex[54]. In organisms such as *B. subtilis,* which lack TatB, TatA is bifunctional and fulfills the roles of both TatA and TatB[32]. After the Tat(AE)BC docking complex has assembled, the signal peptide is inserted into this complex in a hairpin formation that penetrates halfway through the membrane[55]. In *E. coli*, this results in the interaction of the RR-motif of the signal peptide with residues E15 and E105 TatC[49], and of the C-terminal half of the signal peptide with TatB[55]. Subsequently, the precursor protein is committed to further translocation by the Tat machinery[55].

In the next step, an active translocase needs to be assembled for the translocation of the mature Tat substrate to occur. This process requires the pmf and depends on the presence a substrate that is bound by the Tat(AE)BC docking complex[56,57]. Initially, TatA is dispersed through the membrane[57], but the presence of a Tat(AE)BC docking complex with bound substrate leads to TatA recruitment and subsequent oligomerization ($TatA_n$)[58]. The $TatA_n$ forms ring-like structures of variable diameter, including approximately 25 TatA subunits[59,60] that are assembled into $TatA_n$(E)BC complexes[48,57]. During or shortly after membrane passage, the signal peptide is cleaved by signal peptidase, resulting in release of the mature Tat substrate and disassembly of the Tat translocase[61].

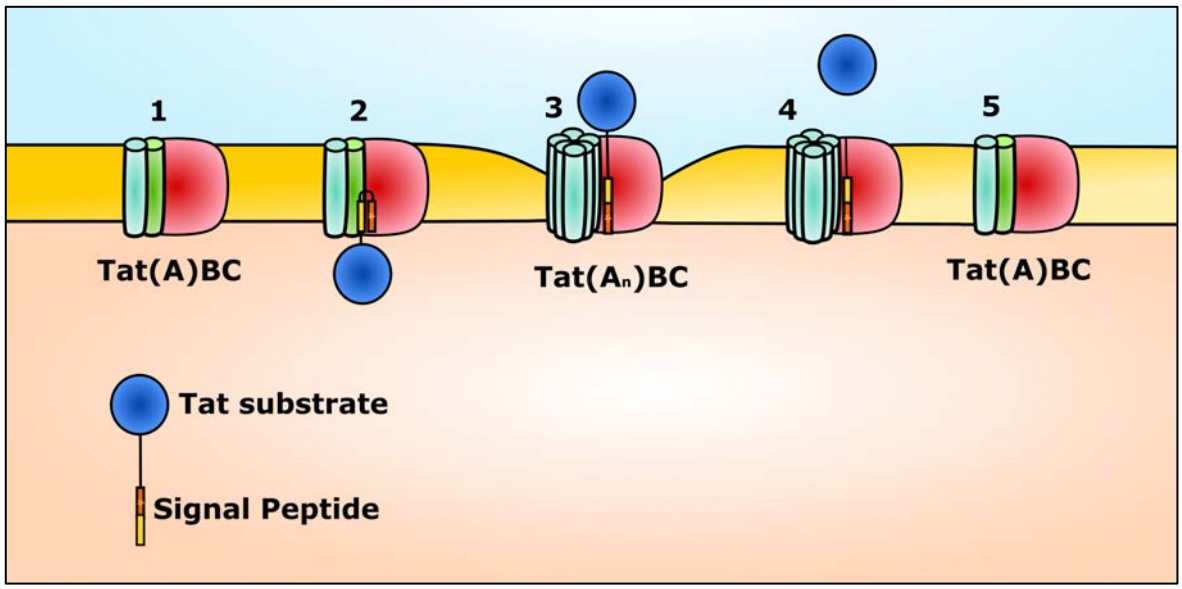

Fig. 2: Schematic representation of the Tat translocation process in *E. coli*. 1. Tat(A)BC receptor complexes are localized in the cytoplasmic membrane, while Tat substrates are properly folded in the cytoplasm. 2. A Tat receptor complex mediates the insertion of a Tat substrate into the membrane in a hairpin conformation. The RR-motif in the signal peptide interacts with TatC, while the C-terminus of the signal peptide interacts with TatB. 3. Additional TatA subunits are recruited to the $TatA_nBC$ docking complex in a pmf-dependent manner. The hairpin in the signal peptide unhinges, and the substrate crosses the membrane by local weakening of the membrane. 5. The protein is released from the signal peptide due to the action of signal peptidases. 6. The TatA complexes dissociate and the Tat(A)BC complex returns to the "relaxed" state. (Modified from [48,63]). Note that TatE serves a similar function as TatA and is, therefore, not depicted in the model.

The mechanism underlying the actual Tat translocation process has been an object of debate over the last two decades. Currently, the main working model for protein translocation *via* Tat hypothesizes that translocation occurs via local weakening of the membrane. In this model, the interaction of $TatA_n$(E)BC with the substrate protein leads to a conformational switch that causes reorientation of the amphipathic helix of TatA. In turn, this would result in a constriction of the membrane width by the transmembrane domain of TatA[47,62], enabling TatC to pull the substrate through the membrane. An alternative model proposes that TatA forms a protein-conducting channel of variable diameter[60]. However, these two models are not mutually exclusive, and both models agree that protein transport through the Tat pathway poses a challenge for the physiology of the cell. Due to the relatively large size of its substrates, it is necessary to safekeep the integrity of the membrane during translocation to avoid the leakage of molecules and ions to the extracytoplasmic space. As a result, the Tat translocase does not form a permanent well-defined porous channel, but rather an "on-demand" channel[57], allowing the cell to maintain its membrane integrity and reduce stress in the absence of translocation[47,50].

Minimal Tat complexes in *B. subtilis*

As indicated above, the Tat machinery of *B. subtilis* is characterized by its minimalistic nature due to the lack of TatB and TatE and the fact that only a TatA and a TatC protein are needed for protein translocation. On the other hand, the Tat system of *B. subtilis* involves three different paralogues of TatA, namely TatAy, TatAd and TatAc, and two different paralogues of TatC, namely TatCy and TatCd (**Fig. 3**), which is a result of gene duplication[43,64]. Further, these Tat subunits can assemble into two different Tat translocases, TatAy-TatCy (TatAyCy) and TatAd-TatCd (TatAdCd). The *tatAyCy* genes form a constitutively expressed operon[65]. This is consistent with the ability of the TatAyCy translocase to transport most Tat substrates of *B. subtilis*[66–68]*.* The known TatAyCy substrates include: 1. the Rieske protein QcrA[41], which contains an iron-sulfur cluster and a disulfide bond; 2. the Dyp-type peroxidase EfeB[69], characterized by the presence of a haem group; and 3. the metallophosphoesterase YkuE[70], containing Mn^{2+} and Zn^{2+} ions in the active site. In contrast, the *tatAdCd* operon is expressed upon phosphate starvation. The respective, TatAdCd translocase specifically transports the phosphodiesterase PhoD. This protein is characterized by the presence of one Fe^{3+} and two Ca^{2+} ions in the active site[71,72]. Currently, the role of the third TatA protein of *B. subtilis,* TatAc, is not completely clear. Transcriptomics studies have shown that *tatAc* is constitutively expressed[65], but biochemical studies have shown that TatAc is not necessary for the translocation of QcrA, EfeB, or PhoD[66], and it does not seem to actively participate in Tat-dependent translocation[73]. However, protein-protein interaction studies by yeast-two hybrid screens have shown that TatAc has high affinity for TatAy and moderate affinity for TatAd[74]. Further, TatAc cannot functionally replace TatAy, although it does compensate for defects in TatAy. These observations suggest that TatAc might have a role in the pore formation or in assisting TatAy[75].

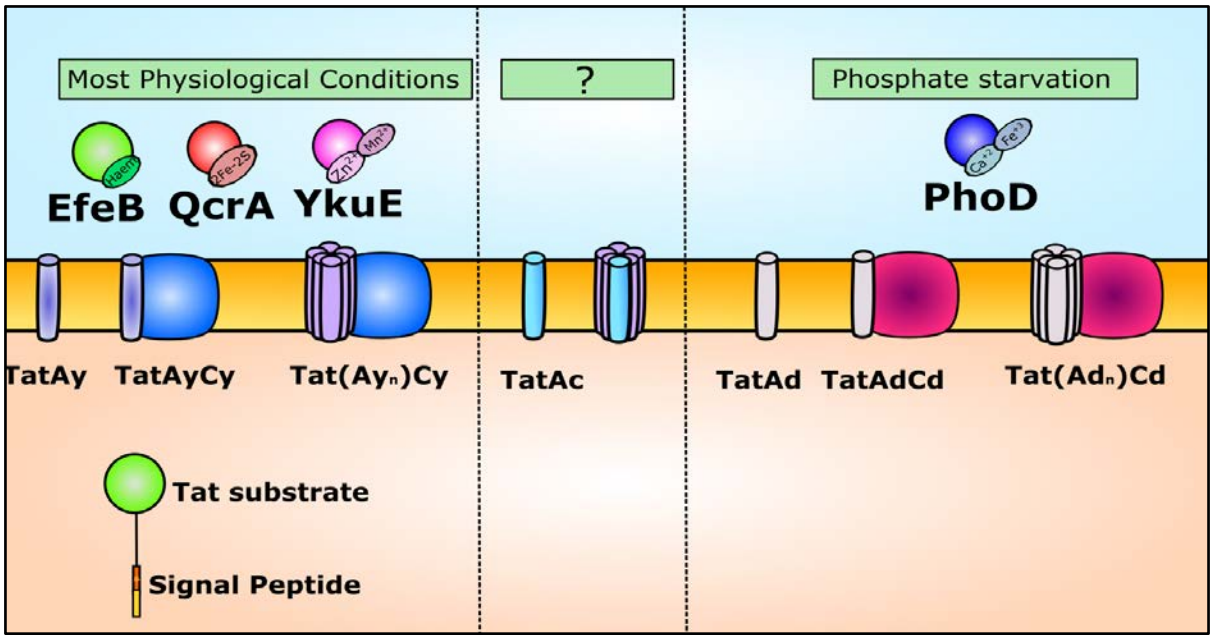

Fig. 3: Minimal Tat translocases of *B. subtilis*. The *B. subtilis* Tat machinery is formed by TatA and TatC proteins. Further, the *B. subtilis* genome encodes three TatA proteins (TatAc, TatAd, and TatAy), and two different TatC proteins (TatCd and TatCy). These proteins can assemble into two different translocases, TatAy-TatCy (TatAyCy) and TatAd-TatCd (TatAdCd). The TatAyCy translocase is active under most physiological conditions and translocates at least three cofactor-containing substrates (EfeB, QcrA, and YkuE). On the other hand, the TatAdCd translocase is active under phosphate starvation and only translocates one cofactor-containing substrate, PhoD. The role of TatAc is not fully understood, but it interacts with both TatAy and TatAd, and it can compensate for specific mutations in TatAy[79].

The Tat pathway has an important role in the lifecycle of *B. subtilis*. Although, not essential for its growth and survival in rich media[76,77], *tatAyCy* deficient strains show a reduced growth rate in iron limited conditions[69]. This correlates with the inability to translocate EfeB across the membrane and into the growth medium. EfeB is part of the EfeUOB transporter complex, which is necessary for the acquisition of ferric and ferrous iron and facilitates ferrous iron utilization in iron-limiting conditions. In addition, EfeB contributes to H_2O_2 detoxification, which relates to the consumption of H_2O_2 during the conversion of ferrous to ferric iron by EfeB[69]. Interestingly, *tatAyCy* mutants also exhibit a severe growth defect in the absence of NaCl from the growth medium. This growth defect is due to an essential EfeB requirement for the acquisition of iron and the detoxification of H_2O_2 under these conditions[78].

Biotechnical Applications of the Tat pathway

The Tat pathway holds great promise for the biotechnology industry due to its ability to transport heterologous proteins incompatible with the Sec pathway. Such proteins may require the insertion of complex cofactors in the cytoplasm, or the activity of cytoplasmic chaperones[80]. Additionally, Sec-incompatible substrates may display rapid folding kinetics[81].

Due to the intrinsic quality control features of the Tat pathway, only properly folded proteins will be exported by this pathway, ensuring a high degree of product homogeneity[40]. The first heterologous protein successfully transported by the Tat pathway to the periplasm of *E. coli* was GFP, fused to the RR-signal peptide of TorA[82,83], a trimethylamine N-oxide reductase with a molybdenum cofactor[84]. This discovery provided the proof-of-principle necessary to start a new chapter in the history of research on the Tat pathway as an alternative facilitator for heterologous protein production. Today, multiple heterologous proteins have been successfully produced via the Tat pathway of *E. coli*, including antibody fragments[40], ribose binding proteins (PBPs)[85], alkaline phosphatase[86], interferon α2b[87], and human growth hormone[88,89].

One of the hurdles for Tat-dependent translocation of heterologous proteins is the limited abundance of translocases in the cell[90]. Consequently, upon high-level substrate overexpression, Tat translocases rapidly become saturated[91], resulting in cell envelope defects[92]. One of the approaches employed to overcome this secretion bottleneck involves overexpression of the Tat machinery together with high-level expression of a heterologous substrate. This has been successfully implemented in *E. coli*, resulting in high product yields without affecting cell viability[93]. In contrast, heterologous protein secretion via the Tat pathway in *B. subtilis* has not been a straightforward matter. In particular, attempts to translocate GFP and various other heterologous proteins via Tat have remained unsuccessful[94] and, to date, the protease subtilisin is the only protein that was successfully re-routed from the Sec pathway into the Tat pathway of *B. subtilis*[95]. Interestingly, upon heterologous expression in *E. coli*, the TatAyCy and TatAdCd translocases are capable of transporting some endogenous Tat substrates[96], as well as GFP, to the extracellular medium[93,97,98]. These findings show that the *Bacillus* Tat translocases have an as yet unidentified proofreading mechanism that is inactive when expressed in *E. coli*. A better understanding of the proofreading mechanism employed by TatAyCy and TatAdCd is therefore key to unlock this pathway for protein production in *B. subtilis*.

Secretion stress responses

During its life cycle, *B. subtilis* is exposed to fluctuations in the environment that can affect the integrity of its cell envelope. As a result, *B. subtilis* has evolved specific stress-responsive mechanisms to protect and maintain the integrity of its cell envelope (**Fig. 4**). The inducing

cues that trigger these mechanisms are not limited to physico-chemical changes in the cells' external milieu, but they also comprise internal parameters, including high-level expression of membrane and secretory proteins. The induction of cell envelope stress-responsive systems results in changes in gene expression in order to repair the damage in the cell envelope and, if possible, to eliminate the stress inducing cue[99]. Importantly, stress induced by high-level secretory protein production, which is termed secretion stress, may set a limit to the production level, especially in the case of heterologous proteins[100]. This may relate to membrane or cell wall perturbations that are triggered by protein aggregation, unfolding, or misfolding[101], and the elicited stress responses usually result in degradation of the overexpressed protein[13].

A common feature that bacteria in general, and *B. subtilis* in particular, employ for adaptation to stressful conditions is the so-called two-component regulatory system (TCS)[102]. Each TCS relies essentially on a histidine kinase (HK) that senses a particular environmental insult and transfers the perceived stimulus to a response regulator protein (RRP) that, in turn, modulates gene expression[103]. In the presence of an inducing cue, the HK auto-phosphorylates a conserved histidine residue. Subsequently, the HK transfers the respective phosphoryl group to the RR, which results in the activation of the effector domain of the RR[102]. The two main TCSs of *B. subtilis* that may be activated upon high-level membrane and secretory protein production are known as CssRS (Control of secretion stress Regulator and Sensor) and LiaRS (Lipid II cycle interfering antibiotic Regulator and Sensor).

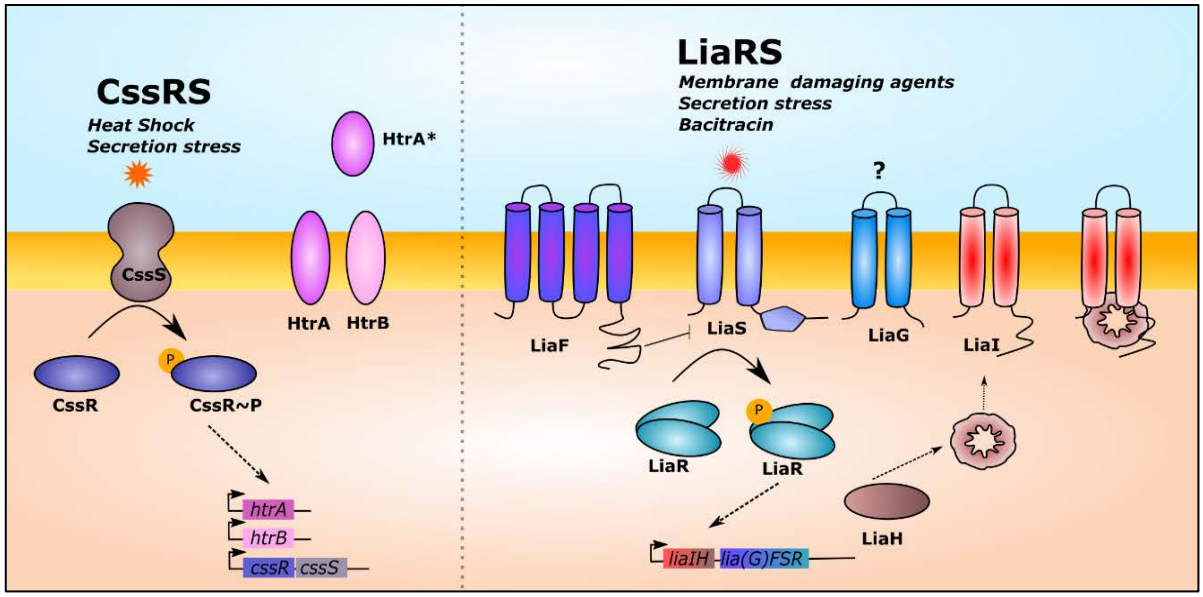

Fig. 4: Secretion stress in *B. subtilis*. Secretion stress in *B. subtilis* leads to the activation of the CssRS and LiaRS stress responses. (A) In response to secretion stress or heat-shock, the two-component stress response system CssRS becomes activated. In the first step the histidine kinase sensor protein CssS, phosphorylates (P) its

cognate response regulator CssR. In turn, this leads to the upregulated synthesis of the membrane proteins HtrA and HtrB. Further, HtrA can also be released into the growth medium, where it has chaperone activity (HtrA*). (B) The LiaRS two-component system is also activated in response to secretion stress or membrane-perturbing conditions. In the presence of such stresses, the negative regulator of the LiaRS response, LiaF, is titrated away, leading to activation of the LiaS sensor and subsequent phosphorylation of its cognate response regulator LiaR. This results in up-regulation of the membrane protein LiaI, and the PspA homologue LiaH. LiaH forms a high-order oligomeric structure, which binds to LiaI, thereby helping to stabilize the membrane.

CssRS - The membrane clean-up crew

The first-identified stress-responsive system involved in the sensing of secretion stress in *B. subtilis* was the CssRS TCS[101,104]. The CssRS system is homologous to the Cpx pathway of *E. coli*, although it evolved adaptations to the Gram-positive bacterial cell envelope structure[101]. In addition to secretion stress, the CssRS system also responds to heat shock, suggesting that it senses protein unfolding[104]. Interestingly, activation of the CssRS system occurs at the extracytoplasmic side of the membrane, and not within the cell [105]. In the presence of a stress stimulus, the precise nature of which still awaits identification, the CssS histidine kinase is activated and phosphorylates the response regulator CssR. In turn, this leads to upregulated expression of the *htrA* and *htrB* genes[101,104]. The two encoded proteases are part of the HtrA family of proteins[106], and they are attached to the cytoplasmic membrane by an N-terminal membrane anchor. Their role consists in maintaining the integrity of the membrane by removing aberrantly folded proteins at the membrane-cell wall interface[107]. HtrA can also be found in the extracytoplasmic space after cleavage of its N-terminal anchor[107]. In its soluble form, HtrA has molecular chaperone properties, indicating that HtrA also helps to prevent protein misfolding related to secretion stress[108]. High-level protein secretion through the Sec pathway frequently results in a production bottleneck due to activation of the CssRS system[101,104] and the subsequent upregulation of HtrA and HtrB[109], which then degrade the target protein[110].

LiaRS - a membrane scaffold during stress

Secretion stress in *B. subtilis* was originally defined as any kind of stress that activates the CssRS TCS[107]. However, multiple studies have indicated that the LiaRS (Lipid II cycle interfering antibiotic Regulator and Sensor) TCS is also highly responsive to secretion stress[111]. The LiaRS system is homologous to the Psp system (phage shock protein) of *E. coli*[112,113]. It was originally identified as part of the *B. subtilis* bacitracin stimulon, and responsible for regulating the expression of the *liaIH-liaGFSR* operons[114]. Further research indicated that expression of these operons is responsive to antimicrobial peptides[115], alkaline

shock[116], ethanol[117], and other cell wall antibiotics that disrupt the lipid II synthesis, such as daptomycin[118 119]. In the absence of stress, the *liaGFSR* genes are constitutively expressed from a weak promoter upstream of *liaG* (P_{liaG}), ensuring that the P_{liaI} promoter remains silenced[120]. Under these conditions, LiaF will inhibit the activity of the histidine kinase LiaS, while LiaH remains dispersed throughout the cytoplasm and LiaI is scattered throughout the membrane[121]. In the presence of a stress-inducing cue, LiaF is titrated away from LiaS, resulting in LiaS activation and subsequent LiaR phosphorylation. The phosphorylated LiaR then binds to the P_{liaI} promoter upstream of *liaIH*, leading to high-level expression of LiaI and LiaH, and readthrough of the *liaIH-liaGFSR* operons. In turn, LiaH forms high-order oligomeric structures and is recruited to static loci by LiaI in the membrane[121]. Given the critical role of LiaF in modulating the activity of LiaRS, this system is also referred to as a three-component system.

LiaH belongs to the PspA/IM30 family, which comprises PspA (Phage Shock Protein A) and VIPP1 (vesicle-inducing protein in plastids 1). Proteins belonging to the PspA/IM30 family are characterized by the ability to form high-molecular weight oligomeric rings that can serve in the stabilization of stressed membranes[122,123]. Interestingly, it has recently been reported by Popp *et al,* that LiaIH is the most effective resistance determinant against activity of the antimicrobial peptide YydF, which causes numerous changes in the membrane permeability and fluidity[124].

Currently, there is no experimental evidence explaining how LiaH relieves membrane stress, but recent studies have shed some light on the mechanism employed by PspA and VIPP1 to maintain the membrane integrity under membrane-disrupting conditions[122,125]. PspA and VIPP1 are involved in sensing so-called stored curvature elastic (SCE) stress. This type of stress is caused by changes in the composition of the membrane, affecting the packing conformation of the lipid bilayer, and resulting in the formation of a curved monolayer[126]. If the SCE is not alleviated, it might result in phase transition of the membrane to a porous state[127]. Consequently, PspA and Vipp1 sense areas in the membrane with increased packing defects as a result of SCE. In turn, this leads to the formation of the afore-mentioned high-order oligomeric structures of PspA and VIPP1 that will stabilize the membrane and prevent transition to a porous state[125]. Remarkably, the Psp system of *E. coli* also shares high functional homology to LiaRS of *B. subtilis*, as this system is also activated in response to damage to the inner membrane caused by misfolded proteins, secretin toxicity[128] heat shock,

and organic solvents[129]. The Psp system of *E. coli* consists of the *pspABCDE* operon, which is regulated by the *pspF* promoter located upstream of the *pspABCDE* locus[130]. However, the Psp response is mediated by the core response that comprises PspA, PspB, PspC, and PspF[131]. In the absence of stress, the PspBC membrane complex actively scans the membrane, and the PspF enhancer-binding protein is repressed by PspA. Under membrane-disrupting conditions or increased SCE[122,128], the PspBC complex recruits PspA, allowing PspF to serve as a transcription factor for induced expression of the *pspABCDE* operon. In case the stress-inducing cue is a misfolded protein, the PspABC complex will interact with the misfolded protein, counteracting its activity and preventing it from damaging the membrane[128]. Alternatively, PspA can directly bind to areas of high SCE via its amphipathic helix, where it forms high order oligomeric structures that help to restore the structure of the membrane[122].

Membrane stress during Tat-dependent protein secretion

Tat-dependent protein translocation is a stressful process for the cell. It requires approximately 10^4 ATP molecules per translocated substrate[132], while the Sec pathway consumes merely 5000 ATP molecules per substrate[133]. Moreover, the cytoplasmic membrane must undergo structural changes to enable the translocation of fully folded Tat substrates while, at the same time, maintaining its integrity to prevent the loss of ions, nutrients and other essential molecules, or the pmf [47,48,63,134]. Additionally, TatA has a high propensity to locally disrupt the membrane, potentially causing an increase in SCE[47,135] which, in *E. coli*, will results in the overexpression of PspA[136]. Interestingly, in *E. coli* it was shown that PspA interacts with the N-terminus of TatA, and this interaction is stabilized by the PspBC complex, although it is not strictly necessary[136]. This process is independent of PspF, suggesting that the Psp response can also sense “intrinsec” stresses. Conversely, high level expression of PspA in response to TatA was shown to lead to a decrease in PspF activity[135]. The mechanism by which PspA alleviates membrane stress during Tat-dependent protein translocation is not fully understood[135]. However, the absence of *pspA* leads to a decrease in the growth rate of *E. coli*, suggesting that PspA is physiologically relevant for survival of *E. coli* during Tat-dependent protein export[136].

Although, the role of PspA in Tat-dependent protein translocation is not precisely understood, it has been demonstrated that PspA overexpression relieves translocation saturation caused by overexpression of natural Tat substrates or heterologous proteins provided with a Tat signal peptide[137]. This effect has also been detected in *Streptomyces*

lividans, where overexpression of PspA resulted in enhanced secretion of Tat substrates[138]. This information indicates that PspA has an active role in maintaining the stability of the membrane during Tat-dependent protein translocation. Notably, PspA is not the only protein of the PspA/IM30 protein family actively involved in Tat-dependent protein translocation. VIPP1 of *Pisum sativum* has also been found to enhance transport via the thylakoidal Tat pathway in a concentration-dependent, saturable manner. However, this stimulation does not seem to depend on VIPP1 directly interacting with any of the Tat components[139].

The Psp and the LiaRS responses actively sense Sec secretion

Continuous monitoring of the cytoplasmic membrane during protein secretion is an essential process to ensure cell viability. In *E. coli* mutations in the Sec components, SecA, SecD and SecF have been shown to activate the Psp response[140]. Similarly, absence of SecDF in *B. cereus* leads to the activation of the LiaRS system[141], indicating that absence of an optimally functional Sec machinery induces envelope stress, most likely as a result of misfolded proteins accumulating in the membrane or particular secretion defects. Interestingly, overproduction of PspA in *S. lividans* has been shown to increase not only Tat-dependent protein secretion, but also Sec-dependent secretion[138]. Together, these findings imply that maintaining membrane integrity has a high priority for the bacterial cell in general, and that proteins like PspA and LiaH serve to meet this requirement in order to allow the cell to mitigate a variety of potentially membrane-disruptive molecular challenges. This applies, especially to the movement of folded and unfolded hydrophilic proteins across the hydrophobic core of the cytoplasmic membrane.

Proteomics as a tool to obtain more information on membrane physiology and secretion stress

Since the advent of the “omics” era, it has been possible to broaden our understanding of the implications of protein translocation on the overall physiology of *B. subtilis* in general, and membrane physiology in particular. Transcriptomics helped to unveil the roles of the LiaRS and CssRS TCSs in dealing with secretion stress[111]. Mass spectrometry-based proteomics approaches have deepened our understanding of Tat-mediated protein secretion in *B. subtilis* by the identification of Tat substrates and Tat pathway-dependent phenotypes[69,70,142,143]. Additionally, proteomics studies have provided valuable insights into the consequences of secretion stress following high-level secretory protein production[107].

While initially two-dimensional gel-based approaches provided semi-quantitative insights in the dynamics of the *B. subtilis* proteome, the more recently applied semi-gel-based or completely gel-free approaches have enabled high-throughput quantitative screening of changes in protein expression levels and post-translational modifications. Importantly, these new approaches have brought about a paradigm shift from relative protein quantification, which already allowed relevant comparisons of protein abundances, to absolute quantification of protein abundance and variations in the proteome. The latter type of information is of critical importance for the development of mathematical models that provide deeper insights into the physiological changes of *B. subtilis* in response to specific environmental cues in general[144], or the high-level production of secretory enzymes in particular. It will be necessary to perform this type of high-quality absolute protein quantifications, in order to develop the next generation of super-secreting *B. subtilis* strains for biotechnological applications through systems biology approaches[145]. However, progress in obtaining absolute quantifications of the membrane proteome of *B. subtilis* was, so far, hindered by the low abundance and highly hydrophobic nature of many integral proteins[146].

Scope of this thesis

In recent years, numerous advances have been made in understanding the impact of protein translocation and secretion on the physiology of *B. subtilis*, as summarized in the Introductory **Chapter 1** of this thesis. However, further progress in this area has been, at least partially, hindered by our limited understanding of relevant processes that take place inside the cytoplasmic membrane. This is due to the hydrophobic nature of the membrane compartment itself, as well as the low abundance and high hydrophobicity of many membrane proteins. The overarching aim of the research described in this PhD thesis was, therefore, to shed more light on the physiology of the membrane under challenging conditions that relate to the translocation of folded proteins via the Tat pathway, or the high-level translocation of difficult-to-produce proteins via the Sec pathway. A better understanding of membrane physiology thus obtained would lay the foundation for the development of next-generation *B. subtilis* production strains. In order to achieve this ambitious objective, it was necessary to employ a combinatory and multidisciplinary approach, where biochemical and “omics” approaches were followed to unveil the behavior of *B. subtilis* cells during Tat- and Sec-mediated translocation.

The aim of the study described in **Chapter 2** was to identify proteins interacting with the minimal Tat translocase TatAyCy. To this end, TatAyCy was overexpressed with the help of a subtilin-inducible promoter and purified by affinity- and size exclusion chromatography. Subsequently, the elution fractions were separated by LDS-PAGE, visualized by Coomassie staining, and the most prominent protein bands were identified by mass spectrometry. This showed that LiaH tightly interacted with the TatAyCy translocase. Furthermore, LiaH was shown to have an active role in TatAyCy translocation, as the absence of this protein led to a decrease in the secretion of the TatAyCy-dependently translocated EfeB protein. In addition, another TatAyCy substrate, namely QcrA, was found to be mislocalized in the absence of LiaH. Together, these observations imply that LiaH has a modulating role in TatAyCy-mediated protein translocation. Importantly, TatAyCy overexpression upon enhanced EfeB production was shown to result in elevated secretion levels of this protein, demonstrating that this successful combinatory approach allows for higher protein secretion levels via the *B. subtilis* Tat pathway.

The studies described in **Chapter 3** of this thesis were focused on obtaining further information on the effects of TatAyCy overexpression on the physiology of *B. subtilis.* To this end, a metabolic labeling and shotgun proteomics approach was implemented. The obtained data show that high-level expression of TatAyCy results in a prolonged vegetative state, which was characterized by the upregulation of proteins involved in motility and chemotaxis, and a concomitant downregulation of proteins involved in biofilm formation, sporulation, and genetic competence.

Conversely, **Chapter 4** describes the physiological effects of the absence of a functional TatAyCy translocase. Previous research had shown that TatAyCy is particularly important for the growth of *B. subtilis* in NaCl-deprived conditions, but the underlying reasons had remained unknown. By employing a transcriptomics approach, it was now shown that the TatAyCy translocase is needed to prevent severe oxidative stress by enabling the translocation of EfeB. Most likely, the activity of EfeB is needed to prevent Fenton-like chemistry through the conversion of ferrous to ferric iron at the expense of H_2O_2. In the absence of EfeB, the resulting oxidative stress precludes nutrient uptake and, consequently, many cells starve to death. A sub-population of these cells manages to survive these conditions by altering their arginine metabolism.

Unlike the studies in **Chapters 2** and **3,** which involved a relative quantification method, the

studies presented in **Chapter 5** were aimed at establishing a workflow for large-scale, absolute membrane proteome quantification. To this end, selected membrane proteins were labeled with a SNAP-tag and used as internal standards for absolute protein quantifications. The thus developed approach was then validated in *B. subtilis* under conditions of hyperosmotic stress, showing that it provides highly sensitive and accurate information on the concentration and abundance of membrane proteins and their complexes.

Chapter 6 presents the first application of the absolute membrane quantification method presented in **Chapter 5** for the quantification of the secretion stress responses in a genome-reduced strain of *B. subtilis* that overproduces the immunodominant *S. aureus* antigen IsaA. The research described in this chapter helped to obtain an unprecedented insight into adaptive changes that occur in the membrane proteome in response to the secretion stress caused by high-level expression of IsaA.

Lastly, **Chapter 7** provides a redefinition of the secretion stress imposed on the *B. subtilis* cell by Tat- or Sec-mediated protein translocation, and it highlights the advantages of employing combined omics and biochemical approaches for this purpose.

References

1. Spizizen, J. Transformation of Biochemically Deficient Strains of *Bacillus* Subtilis By Deoxyribonucleate. *Proc. Natl. Acad. Sci.* **44**, 1072–1078 (1958).
2. Brillard, J. *et al.* The water cycle, a potential source of the bacterial pathogen *Bacillus* cereus. *Biomed Res. Int.* **2015**, (2015).
3. Piewngam, P. & Otto, M. Probiotics to prevent *Staphylococcus aureus* disease? *Gut Microbes* **11**, 94–101 (2020).ƒ
4. Piewngam, P. *et al.* Pathogen elimination by probiotic *Bacillus* via signalling interference. *Nature* **562**, 532–537 (2018).
5. Nichols, D. S., Nichols, P. D. & McMeekin, T. A. Ecology and physiology of psychrophilic bacteria from Antarctic saline lakes and sea-ice. *Sci. Prog.* **78**, 311–347 (1995).
6. Holtmann, G. & Bremer, E. Thermoprotection of *Bacillus subtilis* by exogenously provided glycine betaine and structurally related compatible solutes: Involvement of Opu Transporters. *J. Bacteriol.* **186**, 1683–1693 (2004).
7. Cheng, C. *et al.* Flagellar basal body structural proteins FlhB, FliM, and FliY are required for flagellar-associated protein expression in *Listeria monocytogenes*. *Front. Microbiol.* **9**, 1–11 (2018).
8. Padan, E., Bibi, E., Ito, M. & Krulwich, T. A. Alkaline pH homeostasis in bacteria: New insights. *Biochim. Biophys. Acta - Biomembr.* **1717**, 67–88 (2005).
9. Shioi, J. I., Matsuura, S. & Imae, Y. Quantitative measurements of proton motive force and motility in *Bacillus subtilis*. *J. Bacteriol.* **144**, 891–897 (1980).
10. Harwood, C. R. Introduction to the Biotechnology of *Bacillus*. *Bacillus,* 1–4 (Springer US, 1989).
11. Harwood, C. R. *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnol.* **10**, 247–256 (1992).
12. Contesini, F. J., Melo, R. R. de & Sato, H. H. An overview of *Bacillus* proteases: from production to application. *Crit. Rev. Biotechnol.* **38**, 321–334 (2018).
13. Westers, L., Westers, H. & Quax, W. J. *Bacillus subtilis* as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. *Biochim. Biophys. Acta - Mol. Cell Res.* **1694**, 299–310 (2004).
14. van Dijl, J. M. & Hecker, M. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microb. Cell Fact.* **12**, 3 (2013).
15. Schallmey, M., Singh, A. & Ward, O. P. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* **50**, 1–17 (2004).
16. Gu, Y. *et al.* Advances and prospects of *Bacillus subtilis* cellular factories: From rational design to industrial applications. *Metab. Eng.* **50**, 109–121 (2018).
17. Mergulhão, F. J. M., Summers, D. K. & Monteiro, G. A. Recombinant protein secretion in *Escherichia coli*.

Biotechnol. Adv. **23**, 177–202 (2005).

18. Song, Y., Nikoloff, J. M. & Zhang, D. Improving protein production on the level of regulation of both expression and secretion pathways in *Bacillus subtilis*. *J. Microbiol. Biotechnol.* **25**, 963–977 (2015).

19. Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S. & Van Dijl, J. M. Signal Peptide-Dependent Protein Transport in *Bacillus subtilis* : a Genome-Based Survey of the Secretome Signal Peptide-Dependent Protein Transport in *Bacillus subtilis*. *Mol. Biol. Rev.* **64**, 515–547 (2000).

20. van Roosmalen, M. L. *et al.* Type I signal peptidases of Gram-positive bacteria. *Biochim. Biophys. Acta - Mol. Cell Res.* **1694**, 279–297 (2004).

21. Weiner, J. H. & Rothery, R. A. Bacterial Cytoplasmic Membrane. *Encyclopedia of Life Sciences* (John Wiley & Sons, Ltd, 2007).

22. Freudl, R. Signal peptides for recombinant protein secretion in bacterial expression systems. *Microb. Cell Fact.* **17**, 1–10 (2018).

23. Antelmann, H., Van Dijl, J. M., Bron, S. & Hecker, M. Proteomic survey through secretome of *Bacillus subtilis*. *Methods Biochem. Anal.* **49**, 179–208 (2006).

24. Bonardi, F. *et al.* Probing the SecYEG translocation pore size with preproteins conjugated with sizable rigid spherical molecules. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 7775–7780 (2011).

25. Ulfig, A. & Freudl, R. The early mature part of bacterial twin-arginine translocation (Tat) precursor proteins contributes to TatBC receptor binding. *J. Biol. Chem.* **293**, 7281–7299 (2018).

26. Tsirigotaki, A., De Geyter, J., Šoštarić, N., Economou, A. & Karamanou, S. Protein export through the bacterial Sec pathway. *Nat. Rev. Microbiol.* **15**, 21–36 (2017).

27. Bolhuis, A. *et al.* SecDF of *Bacillus subtilis* , a molecular siamese twin required for the efficient secretion of proteins. *J. Biol. Chem.* **273**, 21217–21224 (1998).

28. Tsukazaki, T. *et al.* Structure and function of a membrane component SecDF that enhances protein export. *Nature* **474**, 235–238 (2011).

29. Tjalsma, H., *et al*. Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev*. **68**, 207–233 (2004).

30. Berks, B. C. A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* **22**, 393–404 (1996).

31. Clark, S. A. & Theg, S. M. A folded protein can be transported across the chloroplast envelope and thylakoid membranes. *Mol. Biol. Cell* **8**, 923–934 (1997).

32. Goosens, V. J., Monteferrante, C. G. & Van Dijl, J. M. The Tat system of Gram-positive bacteria. *Biochim. Biophys. Acta - Mol. Cell Res.* **1843**, 1698–1706 (2014).

33. Settles, A. M. Sec-Independent Protein Translocation by the Maize Hcf106 Protein. *Science.* **278**, 1467–1470 (1997).

34. Sargent, F. *et al.* Overlapping functions of components of a bacterial Sec-independent protein export pathway. *EMBO J.* **17**, 3640–3650 (1998).

35. Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S. & van Dijl, J. M. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* **64**, 515–547 (2000).

36. Kipping, M. *et al.* Structural studies on a twin-arginine signal sequence. *FEBS Lett.* **550**, 18–22 (2003).

37. Fisher, A. C. & Delisa, M. P. A little help from my friends: Quality control of presecretory proteins in bacteria. *J. Bacteriol.* **186**, 7467–7473 (2004).

38. Palmer, T. & Berks, B. C. Moving folded proteins across the bacterial cell membrane. *Microbiology* **149**, 547–556 (2003).

39. Berks, B. C., Palmer, T. & Sargent, F. The Tat protein translocation pathway and its role in microbial physiology. *Adv. Microb. Physiol.* **47**, 187–254 (2003).

40. DeLisa, M. P., Tullman, D. & Georgiou, G. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6115–6120 (2003).

41. Goosens, V. J., Monteferrante, C. G. & Van Dijl, J. M. Co-factor insertion and disulfide bond requirements for twin-Arginine translocase-dependent export of the *Bacillus subtilis* Rieske protein QcrA. *J. Biol. Chem.* **289**, 13124–13131 (2014).

42. Rodrigue, A., Chanal, A., Beck, K., Müller, M. & Wu, L.-F. Co-translocation of a periplasmic enzyme complex by a hitchhiker mechanism through the bacterial Tat pathway. *J. Biol. Chem.* **274**, 13223–13228 (1999).

43. Wu, L. F., Ize, B., Chanal, A., Quentin, Y. & Fichant, G. Bacterial twin-arginine signal peptide-dependent protein translocation pathway: Evolution and mechanism. *J. Mol. Microbiol. Biotechnol.* **2**, 179–189 (2000).

44. Rocco, M. A., Waraho-Zhmayev, D. & DeLisa, M. P. Twin-arginine translocase mutations that suppress folding quality control and permit export of misfolded substrate proteins. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13392–13397 (2012).

45. Jones, A. S. *et al.* Proofreading of substrate structure by the twin-arginine translocase is highly dependent on substrate conformational flexibility but surprisingly tolerant of surface charge and hydrophobicity changes. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 3116–3124 (2016).

46. Hu, Y., Zhao, E., Li, H., Xia, B. & Jin, C. Solution NMR Structure of the TatA component of the twin-arginine protein transport system from Gram-positive bacterium *Bacillus subtilis*. *J. Am. Chem. Soc.* **132**, 15942–15944 (2010).

47. Hou, B., Heidrich, E. S., Mehner-Breitfeld, D. & Brüser, T. The TatA component of the twin-arginine translocation system locally weakens the cytoplasmic membrane of *Escherichia coli* upon protein substrate binding. *J. Biol. Chem.* **293**, 7592–7605 (2018).

48. Hamsanathan, S. & Musser, S. M. The Tat protein transport system: Intriguing questions and conundrums. *FEMS Microbiol. Lett.* **365**, 1–11 (2018).

49. Rollauer, S. E. *et al.* Structure of the TatC core of the twin-arginine protein transport system. *Nature* **492**, 210–214 (2012).

50. Berks, B. C. The Twin-Arginine Protein Translocation Pathway. *Annu. Rev. Biochem.* **84**, 843–864 (2015).

51. Berks, B. C., Sargent, F. & Palmer, T. The Tat protein export pathway. *Mol. Microbiol.* **35**, 260–274 (2000).

52. Frain, K. M., van Dijl, J. M. & Robinson, C. The Twin-Arginine pathway for protein secretion. *EcoSal Plus* **8**, 1–12 (2019).

53. Frain, K. M., Robinson, C. & van Dijl, J. M. Transport of folded proteins by the tat system. *Protein J.* **38**, 377–388 (2019).

54. Alcock, F. *et al.* Assembling the Tat protein translocase. *Elife* **5**, 1–28 (2016).

55. Hamsanathan, S., Anthonymuthu, T. S., Bageshwar, U. K. & Musser, S. M. A Hinged signal peptide hairpin enables Tat-dependent protein translocation. *Biophys. J.* **113**, 2650–2668 (2017).

56. Dabney-Smith, C., Mori, H. & Cline, K. Oligomers of Tha4 organize at the thylakoid Tat translocase during protein transport. *J. Biol. Chem.* **281**, 5476–5483 (2006).

57. Alcock, F. *et al.* Live cell imaging shows reversible assembly of the TatA component of the twin-arginine protein transport system. *Proc. Natl. Acad. Sci. U. S. A.* **110**, (2013).

58. Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M. L. & Robinson, C. TatB and TatC Form a functional and structural unit of the twin-arginine translocase from *Escherichia coli. J. Biol. Chem.* **276**, 20213–20219 (2001).

59. Leake, M. C. *et al.* Variable stoichiometry of the TatA component of the twin-arginine protein transport system observed by in vivo single-molecule imaging. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 15376–15381 (2008).

60. Gohlke, U. *et al.* The TatA component of the twin-arginine protein transport system forms channel complexes of variable diameter. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10482–10486 (2005).

61. Mori, H. & Cline, K. A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid ΔpH/Tat translocase. *J. Cell Biol.* **157**, 205–210 (2002).

62. Brüser, T. & Sanders, C. An alternative model of the twin arginine translocation system. *Microbiol. Res.* **158**, 7–17 (2003).

63. Palmer, T. & Stansfeld, P. J. Targeting of proteins to the twin-arginine translocation pathway. *Mol. Microbiol.* **113**, 861–871 (2020).

64. Simone, D., Bay, D. C., Leach, T. & Turner, R. J. Diversity and evolution of bacterial twin arginine translocase protein, TatC, reveals a protein secretion system that is evolving to fit its environmental niche. *PLoS One* **8**, (2013).

65. Nicolas, P. *et al.* Condition-Dependent Transcriptome Reveals High-Level Regulatory Architecture in *Bacillus subtilis*. *Science.* **335**, 1103–1106 (2012).

66. Jongbloed, J. D. H. *et al.* Two minimal Tat translocases in Bacillus. *Mol. Microbiol.* **54**, 1319–1325 (2004).

67. Jongbloed, J. D. H. *et al.* TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. *J. Biol. Chem.* **275**, 41350–41357 (2000).

68. Widdick, D. A., Eijlander, R. T., van Dijl, J. M., Kuipers, O. P. & Palmer, T. A Facile Reporter System for

the Experimental Identification of Twin-Arginine Translocation (Tat) Signal Peptides from All Kingdoms of Life. *J. Mol. Biol.* **375**, 595–603 (2008).

69. Miethke, M., Monteferrante, C. G., Marahiel, M. A. & van Dijl, J. M. The *Bacillus subtilis* EfeUOB transporter is essential for high-affinity acquisition of ferrous and ferric iron. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833**, 2267–2278 (2013).

70. Monteferrante, C. G., Miethke, M., Van Der Ploeg, R., Glasner, C. & Van Dijl, J. M. Specific targeting of the metallophosphoesterase YkuE to the Bacillus cell wall requires the twin-arginine translocation system. *J. Biol. Chem.* **287**, 29789–29800 (2012).

71. Eder, S., Shi, L., Jensen, K., Yamane, K. & Hulett, F. M. A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a Pho regulon gene, phoD. *Microbiology* **142**, 2041–2047 (1996).

72. Jongbloed, J. D. H. *et al.* TatC Is a specificity determinant for protein secretion via the twin-arginine Translocation Pathway. *J. Biol. Chem.* **275**, 41350–41357 (2000).

73. Eijlander, R. T., Jongbloed, J. D. H. & Kuipers, O. P. Relaxed specificity of the *Bacillus subtilis* TatAdCd translocase in Tat-dependent protein secretion. *J. Bacteriol.* **91**, 196–202 (2009).

74. Monteferrante, C. G. *et al.* Mapping the twin-arginine protein translocation network of *Bacillus subtilis*. *Proteomics* **13**, 800–811 (2013).

75. Goosens, V. J., De-San-Eustaquio-Campillo, A., Carballido-López, R. & van Dijl, J. M. A Tat ménage à trois - The role of *Bacillus subtilis* TatAc in twin-arginine protein translocation. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 2745–2753 (2015).

76. van Der Ploeg, R. *et al.* Salt sensitivity of minimal twin arginine translocases. *J. Biol. Chem.* **286**, 43759–43770 (2011).

77. Eijlander, R. T., Kolbusz, M. A., Berendsen, E. M. & Kuipers, O. P. Effects of altered TatC proteins on protein secretion efficiency via the twin-arginine translocation pathway of *Bacillus subtilis*. *Microbiology* **155**, 1776–1785 (2009).

78. van der Ploeg, R. *et al.* Environmental salinity determines the specificity and need for tat-dependent secretion of the YwbN protein in *Bacillus subtilis*. *PLoS One* **6**, (2011).

79. Goosens, V. J., De-San-Eustaquio-Campillo, A., Carballido-López, R. & van Dijl, J. M. A Tat ménage à trois - The role of *Bacillus subtilis* TatAc in twin-arginine protein translocation. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 2745–2753 (2015).

80. Graubner, W., Schierhorn, A. & Brüser, T. DnaK plays a pivotal role in Tat targeting of CueO and functions beside SlyD as a general Tat signal binding chaperone. *J. Biol. Chem.* **282**, 7116–7124 (2007).

81. Palmer, T. & Berks, B. C. The twin-arginine translocation (Tat) protein export pathway. *Nat. Rev. Microbiol.* **10**, 483–496 (2012).

82. Thomas, J. D., Daniel, R. A., Errington, J. & Robinson, C. Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. *Mol. Microbiol.* **39**, 47–53 (2001).

83. Santini, C. L. *et al.* Translocation of Jellyfish Green Fluorescent Protein via the Tat System of *Escherichia coli* and Change of Its Periplasmic Localization in Response to Osmotic Up-shock. *J. Biol. Chem.* **276**, 8159–8164 (2001).

84. Santini, C. L. *et al.* A novel Sec-independent periplasmic protein translocation pathway in *Escherichia coli*. *EMBO J.* **17**, 101–112 (1998).

85. Pradel, N. *et al.* Influence of tat mutations on the ribose-binding protein translocation in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **306**, 786–791 (2003).

86. Masip, L. *et al.* An Engineered Pathway for the Formation of Protein Disulfide Bonds. *Science.* **303**, 1185–1189 (2004).

87. Alanen, H. I. *et al.* Efficient export of human growth hormone, interferon α2b and antibody fragments to the periplasm by the *Escherichia coli* Tat pathway in the absence of prior disulfide bond formation. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 756–763 (2015).

88. Browning, D. F. *et al. Escherichia coli* "TatExpress" strains super-secrete human growth hormone into the bacterial periplasm by the Tat pathway. *Biotechnol. Bioeng.* **114**, 2828–2836 (2017).

89. Guerrero Montero, I. *et al. Escherichia coli* "TatExpress" strains export several g/L human growth hormone to the periplasm by the Tat pathway. *Biotechnol. Bioeng.* **116**, 3282–3291 (2019).

90. Jack, R. L., Sargent, F., Berks, B. C., Sawers, G. & Palmer, T. Constitutive expression of *Escherichia coli* tat genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. *J. Bacteriol.* **183**, 1801–1804 (2001).

91. Barrett, C. M. L., Ray, N., Thomas, J. D., Robinson, C. & Bolhuis, A. Quantitative export of a reporter protein, GFP, by the twin-arginine translocation pathway in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **304**, 279–284 (2003).

92. Izet, B. *et al.* Novel phenotypes of *Escherichia coli* tat mutants revealed by global gene expression and phenotypic analysis. *J. Biol. Chem.* **279**, 47543–47554 (2004).

93. Branston, S. D., Matos, C. F. R. O., Freedman, R. B., Robinson, C. & Keshavarz-Moore, E. Investigation of the impact of Tat export pathway enhancement on E. coli culture, protein production and early stage recovery. *Biotechnol. Bioeng.* **109**, 983–991 (2012).

94. van der Ploeg, R. *et al.* High-salinity growth conditions promote tat-independent secretion of tat substrates in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **78**, 7733–7744 (2012).

95. Kolkman, M. A. B. *et al.* The twin-arginine signal peptide of *Bacillus subtilis* YwbN can direct either Tat- or Sec-dependent secretion of different cargo proteins: Secretion of active subtilisin via the B. subtilis Tat pathway. *Appl. Environ. Microbiol.* **74**, 7507–7513 (2008).

96. Mendel, S. *et al.* The *Escherichia coli* TatABC System and a *Bacillus subtilis* TatAC-type System Recognise Three Distinct Targeting Determinants in Twin-arginine Signal Peptides. *J. Mol. Biol.* **375**, 661–672 (2008).

97. Albiniak, A. M. *et al.* High-level secretion of a recombinant protein to the culture medium with a *Bacillus subtilis* twin-arginine translocation system in *Escherichia coli*. *FEBS J.* **280**, 3810–3821 (2013).

98. Barnett, J. P. *et al.* The twin-arginine translocation (Tat) systems from *Bacillus subtilis* display a conserved mode of complex organization and similar substrate recognition requirements. *FEBS J.* **276**, 232–243 (2009).

99. Jordan, S., Hutchings, M. I. & Mascher, T. Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol. Rev.* **32**, 107–146 (2008).

100. Westers, H. *et al.* The Bacillus secretion stress response is an indicator for α-amylase production levels. *Lett. Appl. Microbiol.* **39**, 65–73 (2004).

101. Hyyryläinen, H. L. *et al.* A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. *Mol. Microbiol.* **41**, 1159–1172 (2001).

102. Stock, A. M., Robinson, V. L. & Goudreau, P. N. Two-Component Signal Transduction. *Annu. Rev. Biochem.* **69**, 183–215 (2000).

103. Groisman, E. A. Feedback Control of Two-Component Regulatory Systems. *Annu. Rev. Microbiol.* **70**, 103–124 (2016).

104. Darmon, E. *et al.* A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. *J. Bacteriol.* **184**, 5661–5671 (2002).

105. Marciniak, B. C., Trip, H., van-der Veek, P. J. & Kuipers, O. P. Comparative transcriptional analysis of *Bacillus subtilis* cells overproducing either secreted proteins, lipoproteins or membrane proteins. *Microb. Cell Fact.* **11**, 1–13 (2012).

106. Hansen, G. & Hilgenfeld, R. Architecture and regulation of HtrA-family proteins involved in protein quality control and stress response. *Cell. Mol. Life Sci.* **70**, 761–775 (2013).

107. Antelmann, H. *et al.* The extracellular proteome of *Bacillus subtilis* under secretion stress conditions. *Mol. Microbiol.* **49**, 143–156 (2003).

108. Yan, S. & Wu, G. Proteases HtrA and HtrB for α-amylase secreted from *Bacillus subtilis* in secretion stress. *Cell Stress Chaperones* **24**, 493–502 (2019).

109. Neef, J., Bongiorni, C., Goosens, V. J., Schmidt, B. & van Dijl, J. M. Intramembrane protease RasP boosts protein production in Bacillus. *Microb. Cell Fact.* **16**, 1–9 (2017).

110. Yan, S. & Wu, G. Bottleneck in secretion of α-amylase in *Bacillus subtilis*. *Microb. Cell Fact.* **16**, 1–8 (2017).

111. Hyyryläinen, H. L., Sarvas, M. & Kontinen, V. P. Transcriptome analysis of the secretion stress response of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **67**, 389–396 (2005).

112. Rietkötter, E., Hoyer, D. & Mascher, T. Bacitracin sensing in *Bacillus subtilis*. *Mol. Microbiol.* **68**, 768–785 (2008).

113. Manganelli, R. & Gennaro, M. L. Protecting from envelope stress: variations on the Phage-shock-protein theme. *Trends Microbiol.* **25**, 205–216 (2017).

114. Mascher, T., Margulis, N. G., Wang, T., Ye, R. W. & Helmann, J. D. Cell wall stress responses in *Bacillus subtilis*: The regulatory network of the bacitracin stimulon. *Mol. Microbiol.* **50**, 1591–1604 (2003).

115. Pietiäinen, M. *et al.* Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems. *Microbiology* **151**, 1577–1592 (2005).

116. Wiegert, T., Homuth, G., Versteeg, S. & Schumann, W. Alkaline shock induces the *Bacillus subtilis* σ^W regulon. *Mol. Microbiol.* **41**, 59–71 (2001).

117. Petersohn, A. *et al.* Global analysis of the general stress response of *Bacillus subtilis*. *J. Bacteriol.* **183**, 5617–5631 (2001).

118. Hachmann, A.-B., Angert, E. R. & Helmann, J. D. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. *Antimicrob. Agents Chemother.* **53**, 1598–1609 (2009).

119. Mascher, T., Zimmer, S. L., Smith, T. A. & Helmann, J. D. Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrob. Agents Chemother.* **48**, 2888–2896 (2004).

120. Jordan, S., Junker, A., Helmann, J. D. & Mascher, T. Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: Identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. *J. Bacteriol.* **188**, 5153–5166 (2006).

121. Domínguez-Escobar, J. *et al.* Subcellular localization, interactions and dynamics of the phage-shock protein-like Lia response in *Bacillus subtilis*. *Mol. Microbiol.* **92**, 716–732 (2014).

122. McDonald, C., Jovanovic, G., Wallace, B. A., Ces, O. & Buck, M. Structure and function of PspA and Vipp1 N-terminal peptides: Insights into the membrane stress sensing and mitigation. *Biochim. Biophys. Acta - Biomembr.* **1859**, 28–39 (2017).

123. Thurotte, A., Brüser, T., Mascher, T. & Schneider, D. Membrane chaperoning by members of the PspA/IM30 protein family. *Commun. Integr. Biol.* **10**, e1264546 (2017).

124. Popp, P. F., Benjdia, A., Strahl, H., Berteau, O. & Mascher, T. The epipeptide YydF intrinsically triggers the cell envelope stress Response of *Bacillus subtilis* and causes severe membrane perturbations. *Front. Microbiol.* **11**, 1–13 (2020).

125. McDonald, C., Jovanovic, G., Ces, O. & Buck, M. Membrane stored curvature elastic stress modulates recruitment of maintenance proteins PspA and VIPP1. *MBio* **6**, 1–10 (2015).

126. Booth, P. J. & Curnow, P. Current Opinion in Structural Biology Folding scene investigation: membrane proteins. **19**, 8–13 (2009).

127. Attard, G. S., Templer, R. H., Smith, W. S., Hunt, A. N. & Jackowski, S. Modulation of CTP:phosphocholine cytidylyltransferase by membrane curvature elastic stress. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9032–9036 (2000).

128. Srivastava, D., Moumene, A., Flores-Kim, J. & Darwin, A. J. Psp stress response proteins form a complex with mislocalized secretins in the *Yersinia enterocolitica* cytoplasmic membrane. *MBio* **8**, 1–15 (2017).

129. Model, P. & Jovanovic, G. MicroReview The *Escherichia coli* Phage-shock-protein (Psp) operon. **24**, 255–261 (1997).

130. Joly, N. *et al.* Managing membrane stress: The phage shock protein (Psp) response, from molecular mechanisms to physiology. *FEMS Microbiol. Rev.* **34**, 797–827 (2010).

131. Huvet, M. *et al.* The evolution of the phage shock protein response system: Interplay between protein function, genomic organization, and system function. *Mol. Biol. Evol.* **28**, 1141–1155 (2011).

132. Alder, N. N. & Theg, S. M. Energetics of protein transport across biological membranes: A study of the thylakoid ΔpH-dependent/cpTat pathway. *Cell* **112**, 231–242 (2003).

133. Driessen, A. J. Precursor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive force. *EMBO J.* **11**, 847–853 (1992).

134. Teter, S. A. & Theg, S. M. Energy-transducing thylakoid membranes remain highly impermeable to ions during protein translocation. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 1590–1594 (1998).

135. Heidrich, E. S. & Brüser, T. Evidence for a second regulatory binding site on PspF that is occupied by the C-terminal domain of PspA. *PLoS One* **13**, 1–20 (2018).

136. Mehner, D., Osadnik, H., Lünsdorf, H. & Brüser, T. The Tat system for membrane translocation of folded proteins recruits the membrane-stabilizing Psp machinery in *Escherichia coli*. *J. Biol. Chem.* **287**, 27834–27842 (2012).

137. Delisa, M. P., Lee, P., Palmer, T. & Georgiou, G. Phage Shock Protein PspA of *Escherichia coli* Relieves Saturation of Protein Export via the Tat Pathway. *Microbiology* **186**, 366–373 (2004).

138. Vrancken, K. *et al.* PspA overexpression in *Streptomyces lividans* improves both Sec- and Tat-dependent protein secretion. *Appl. Microbiol. Biotechnol.* **73**, 1150–1157 (2007).

139. Lo, S. M. & Theg, S. M. Role of vesicle-inducing protein in plastids 1 in cpTat transport at the thylakoid. *Plant J.* **71**, 656–668 (2012).

140. Jones, S. E., Lloyd, L. J., Tan, K. K. & Buck, M. Secretion defects that activate the phage Shock response of *Escherichia coli*. *J. Bacteriol.* **185**, 6707–6711 (2003).

141. Vörös, A. *et al.* SecDF as part of the sec-translocase facilitates efficient secretion of *Bacillus cereus* toxins and cell wall-associated proteins. *PLoS One* **9**, (2014).

142. Goosens, V. J. *et al.* Novel twin-arginine translocation pathway-dependent phenotypes of *Bacillus subtilis* unveiled by quantitative proteomics. *J. Proteome Res.* **12**, 796–807 (2013).

143. Stewart, D. *et al.* Omics Technologies Used in Systems Biology. in *Systems Biology in Toxicology and Environmental Health* 57–83 (Elsevier, 2015).

144. Maaß, S. *et al.* Highly precise quantification of protein molecules per cell during stress and starvation responses in *Bacillus subtilis*. *Mol. Cell. Proteomics* **13**, 2260–2276 (2014).

145. Muntel, J. *et al.* Comprehensive absolute quantification of the cytosolic proteome of *Bacillus subtilis* by data independent, parallel fragmentation in liquid chromatography/mass spectrometry (LC/MSE). *Mol. Cell. Proteomics* **13**, 1008–1019 (2014).

146. Antelo-Varela, M. *et al.* Ariadne's Thread in the analytical labyrinth of membrane proteins: Integration of targeted and shotgun proteomics for global absolute quantification of membrane proteins. *Anal.*

Chem. **91**, 11972–11980 (2019).